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Properties of Cholesterol Dissimilation by *Rhodococcus equi*

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ABSTRACT

The identification of serum cholesterol as a risk factor in the development of coronary heart disease has resulted in attempts to reduce dietary cholesterol intake. As a result, the removal of cholesterol from food products has become a challenging research objective. We report here the loss of 40% of the cholesterol found in egg yolk preparations following treatment with sonicated extracts of *Rhodococcus equi*. Extracts of *R. equi* 21107 and *R. equi* 33706 removed 3.33% and 3.09% of the cholesterol from egg yolk per min per mg of crude enzyme protein, respectively. Incubation of fresh cream with *R. equi* 33706 extracts resulted in only a 2.4% reduction in cholesterol content (rate of reduction: 0.35% cholesterol per min per mg of protein). Cholesterol degradation by *R. equi* 33706 had an optimum temperature of 40°C and an optimum pH of 8.0, and there was no apparent requirement for divalent metal ions. Approximately 44% of enzyme activity was lost after a 60 min exposure at 60°C. Thin layer chromatographic analysis of cholesterol degradation products revealed only a few steroid-like compounds, primarily 4-cholesten-3-one and 1,4-cholestadiene-3-one.

The link between high serum cholesterol levels and coronary heart disease (CHD) had been strongly suggested by results of epidemiological, clinical, and animal research. Evidence that the diet influences blood cholesterol levels and CHD was reviewed by Grundy et al. (*4*). Dietary intervention trials in humans uniformly indicated a positive trend toward decreased CHD risk with decreased cholesterol intake. The National Cholesterol Education Program Coordinating Board, the American Heart Association, the U.S. Department of Agriculture, and the Department of Health and Human Services have all been consistent in recommending that the daily cholesterol intake in the American diet be limited to 300 mg or less. As a result of these reports, reduction of cholesterol in food items relatively rich in cholesterol (dairy foods, eggs) has become a challenging industrial problem.

Removal or reduction of the cholesterol content of milk and eggs is attractive since cholesterol occurs in relatively high amounts in these products. The cholesterol content of eggs is approximately 250 mg per yolk and that of whole milk 34 mg per cup (*9*).

The following study was undertaken to determine the effectiveness of reducing the cholesterol content of dairy and egg products by means of cholesterol metabolizing systems of bacteria. A number of organisms have been shown to degrade cholesterol, however only a few do so without accumulating any steroid intermediates (*1,2,7,8*). In this study, strains of the genera *Rhodococcus, Nocardium, Brevibacterium,* and *Microbacterium* were tested for their ability to degrade cholesterol. Sonicated extracts prepared from organisms showing the highest cholesterol degrading activity were tested for their ability to reduce the cholesterol content of egg yolk and milk cream preparations.

This material was presented in part at the 84th Annual Meeting of the American Dairy Science Association, 31 July — 4 August, 1989, Lexington, KY.

MATERIALS AND METHODS

Microorganisms and growth conditions

Rhodococci, brevibacteria, and microbacteria used in this study were obtained from the American Type Culture Collection (Rockville, MD). *Nocardia* strains were from the Northern Regional Research Center, USDA (Peoria, IL). The test organisms were maintained in Brain Heart Infusion Broth (Difco Laboratories, Detroit, MI).

Microbial degradation of cholesterol

Bacterial cells were grown at 30°C in Bennet's medium (per liter: yeast extract 0.2 g, beef extract 0.2 g, neopeptone 0.4 g, and glucose 0.02 g) supplemented with 0.5% polyoxyethanyl-cholesteryl sebacate (Sigma Chemical Company, St. Louis, MO), using an orbital incubator shaker set at 250 rpm. Microbial strains showing high cholesterol metabolizing activity were selected and grown in BHI broth supplemented with cholesterol (Sigma Chemical Co., St. Louis, MO) at 125 µg/ml (dissolved in n-propanol) and incubated at 30°C in an orbital incubator shaker.

Resting cells of test organisms were prepared by growing the strains in BHI broth with and without free cholesterol. After 3 d at 30°C, cells were harvested by centrifugation at 12,000 x g and 4°C, washed twice and resuspended in 50 mM potassium phosphate buffer (pH 7.4) supplemented with cholesterol stock solution in propanol at a final concentration of 125 µg/ml and incubated at 30°C in a rotary incubator shaker.

Preparation of cell extracts by sonication

Cells were grown in BHI-cholesterol medium for 3 d at 30°C, washed twice with 50 mM potassium phosphate buffer (pH 7.4) and resuspended in buffer. Sonic disruption was repeated 4 times

for 30 s with intermittent cooling on ice. The cell debris was removed by centrifugation and the supernatant was used as the source of the cholesterol degrading system. Protein was determined by the method of Lowry (*6*).

Cell free extracts were tested for cholesterol degrading activity by mixing 200 µl of the extract in 1.6 ml of 50 mM phosphate buffer (pH 7.4) containing 200 µl of cholesterol (2 mg/ml) in n-propanol. Reactions were carried out for 60 min at 37°C and stopped by extraction with 4 ml of ethyl acetate. Aliquots (1 ml) of the ethyl acetate layer were assayed for cholesterol content. Cholesterol degrading activity was calculated as µg cholesterol degraded per min per mg of protein.

Reduction of cholesterol in egg yolk and milkfat

Egg yolk substrate was prepared by removing yolk from raw eggs under sterile conditions and diluting with 50 mM potassium phosphate buffer (pH 7.4). The final concentration of cholesterol in the egg yolk substrate was approximately 60 µg/ml. Preparation of butterfat as a substrate included a 9:1 (v/v) dilution of dairy cream with 500 mM potassium phosphate buffer (pH 7.4). Cholesterol concentration of the diluted cream was 90 µg/ml. The effect of cell free extracts on cholesterol in egg yolk and cream preparations was determined by incubating 1.0 ml of extract with 1.0 ml substrate for 60 min at 37°C.

Determination of cholesterol

Cholesterol was determined by the ferric chloride method (*11*). Test samples were extracted with an equal volume of ethyl acetate; after separation of phases by centrifugation at 6000 x g for 3 min, aliquots (1.0 ml) of the ethyl acetate layer were evaporated to dryness. Residues were dissolved in 3 ml of the ferric chloride reagent (ferric chloride dissolved at 70 mg/100 ml in acetic acid) and 2 ml of concentrated H_2SO_4 were added with vigorous vortexing to obtain thorough mixing. Reaction mixtures were allowed to cool to room temperature and absorbance was read at 560 nm. Residual cholesterol content of egg yolk and milk cream preparations was determined with 100 µl aliquots, without solvent extraction.

Thin layer chromatography

Cholesterol degradation by growing cells, resting cells, and cell free extracts was also followed by thin layer chromatography (TLC). Ethyl acetate extracts were analyzed on SIL G-25 silica gel plates in a chloroform: ethyl acetate (95:5 v/v) solvent system at room temperature. Cholesterol and its steroid degradation products were detected by spraying with a phosphoric acid (8.5%)-copper sulfate (10%) reagent and heating at 130°C for 5 min. Fluorescent spots were observed under UV light.

Materials

All chemicals, solvents and reagents were commercial products of the highest analytical purity. Microbiological medium components were purchased from Difco Laboratories, Detroit, MI.

RESULTS AND DISCUSSION

Microbial dissimilation of cholesterol

The extent of cholesterol dissimilation after growing the test organisms in Bennet's medium supplemented with soluble cholesterol (polyoxyethanyl-cholesteryl sebacate) for up to 7 d is shown in Table 1. Approximately 80% of the cholesterol supplied was degraded by 10 of the 27 cultures tested after 7 d of growth. However, only four of these organisms degraded 30% or more of the cholesterol supplied after 3 d of growth.

TABLE 1. *Percentage of cholesterol degraded during the growth of various microorganisms.*

Organism	Growth Period (Days)[a] 3	5	7
Rhodococcus equi ATCC 33702	0	0	13.0
Rhodococcus equi ATCC 33703	41.5	82.4	85.4
Rhodococcus equi ATCC 33704	26.2	58.0	77.4
Rhodococcus equi ATCC 33705	31.0	73.3	85.2
Rhodococcus equi ATCC 33706	48.5	78.7	86.2
Rhodococcus equi ATCC 21107	32.3	82.4	86.9
Rhodococcus equi ATCC 21329	0	0	0
Rhodococcus equi ATCC 6939	11.6	58.1	77.3
Rhodococcus equi ATCC 13556	0.9	10.3	15.3
Rhodococcus equi ATCC 13557	0	40.6	74.3
Rhodococcus equi ATCC 21280	0	0	0
Rhodococcus equi ATCC 21690	2.8	61.9	80.5
Rhodococcus equi ATCC 7698	19.5	58.1	80.8
Rhodococcus equi ATCC 7699	20.9	65.2	80.6
Brevibacterium casein ATCC 35513	4.0	10.0	10.0
Brevibacterium linens ATCC 9172	0	0	0
Brevibacterium lipolyticum NRRL B4202	3.6	32.6	55.0
Microbacterium lacticum ATCC 8180	7.5	7.2	7.1
Microbacterium lacticum ATCC 8181	5.9	6.2	6.2
Nocardia corallinia NRRL B3906	0	0	0
Norcardia opaca NRRL B3311	0	0	0
Nocardia erythropolis NRRL B1532	0	6.1	31.2
Nocardia corallina NRRL B5476	0	0	0
Nocardia minima NRRL B5477	0	0	0
"Amycolatopsis-like" strain NRRL B16284	0	23.5	30.0
Nocardia rubra NRRL B685	2.7	5.9	5.9
Nocardia globerula NRRL B1306	0	0	0

[a]Growth conditions are described in Materials and Methods.

TLC analysis of ethyl acetate extracts from cholesterol dissimilation studies showed that actively growing cultures degraded cholesterol without accumulating appreciable amounts of steroid intermediate products. The chromatograms of samples extracted in a time study with *Rhodococcus* strains revealed 4-cholesten-3-one to be the first product of cholesterol degradation confirming the presence of an oxidative pathway in this organism (*14*). The rate of cholesterol degradation by rhodococci was generally higher than that shown by *Nocardia* strains as evidenced by the TLC analysis of reaction mixtures for residual cholesterol. The higher rate of cholesterol degradation by *Rhodococcus* strains was already noted during initial growth studies of the test organisms (Table 1). Figure 1 shows that *R. equi* 33706 extracts nearly completely depleted the cholesterol substrate after 4 h of incubation. In contrast, cell-free extracts of *N. erythropolis* B1532 required more than 7 h for the complete degradation of cholesterol supplied. In the absence of enzyme extracts, cholesterol degradation was not observed.

TLC analysis of ethyl acetate extracts of resting cell suspensions of *R. equi* 33706 and *N. erythropolis* B1532 showed the accumulation of several steroid intermediates

(Fig. 2). In quantitative terms, the initial cholesterol concentration of the *R. equi* 33706 reaction system decreased from 250 μg/ml to 8.1 μg/ml after 2 h by cells pregrown without cholesterol. Cells pregrown in the presence of cholesterol reduced the amount of cholesterol below the limit of detectability of the ferric chloride test within 2 h of incubation. These results were in agreement with that previously reported by others (*1,14*), confirming that actively growing cultures of *R. equi* metabolize cholesterol without accumulating large amounts of steroid intermediates. However, if the rate of cellular metabolism is decreased as in the case of resting cells, certain intermediate degradation products (4-cholesten-3-one, 1,4-cholestadiene-3-one, and other unidentified steroid intermediates) can be detected. These results also implied that the expression of the cholesterol metabolizing system of *R. equi* is, at least in part, constitutively controlled since cells grown initially without cholesterol also showed high levels of cholesterol degrading activity. The partially constitutive control of this activity was also seen when *R. equi* 33706 was grown in minimal medium (g per liter: $(NH_4)_2SO_4$, 0.58; K_2HPO_4, 0.25 ; $MgSo_4.7H_2O$, 0.25; $FeSo_4.H_2O$, 0.001; yeast extract, 5.0) with and without cholesterol. Both types of cell extracts degraded cholesterol; however, the rate of substrate dissimilation by the extract from cells grown without cholesterol amounted only to 58% of the activity observed with the extract from cells grown in the presence of cholesterol.

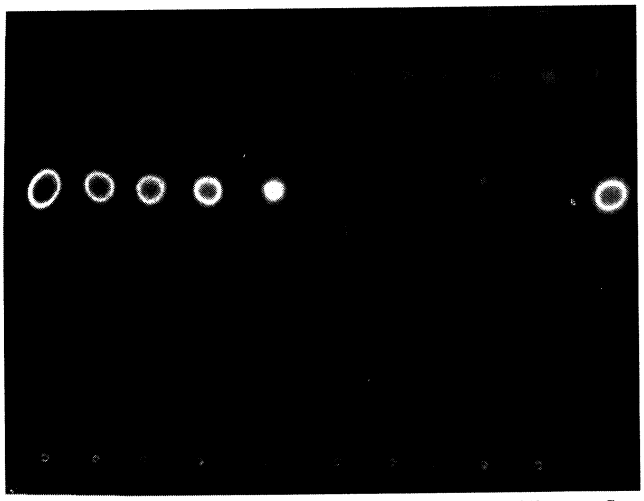

Figure 1. *Cholesterol degradation by cell free extracts of* R. equi *33706. Reaction mixture: 16 ml enzyme extract and 2.0 ml cholesterol in n-propanol (4.0 mg/ml), were mixed and incubated at 37°C. Samples (1 ml) were taken at the times indicated and extracted with 2.5 ml of ethyl acetate. The solvent phases were reduced to a standard volume and spotted on Silica Gel Plates. Solvent System: Chloroform-Ethyl Acetate (95:5, v/v). Detection: $CuSO_4$-H_3PO_4 (10% - 8.5%) Reagent. C: cholesterol standard. (Note: elapsed time between substrate addition and sampling was approximately 60 s resulting in fast migrating cholesterol degradation products already visible at "0 min").*

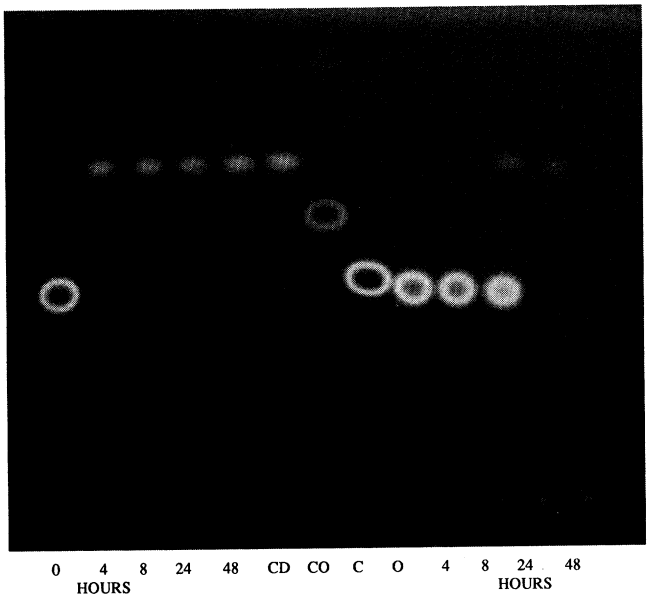

Figure 2. *Cholesterol degradation by resting cells of* R. equi *33706 and* N. erythropolis *B1532. After 72 h of growth, cells were suspended in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.4) with 250 μg/ml of cholesterol. Aliquots were taken at the times indicated and treated as described in* Materials and Methods. *Standards: C, cholesterol; CO, 4-cholesten-3-one; CD, 1,4-cholestadiene-3-one.*

Effect of cholesterol source on substrate degradation

Strains of *R. equi* most efficient in degrading cholesterol (50% or more of cholesterol supplied) after 5 d of growth were studied in more detail. Sonicated extracts of 72-h cultures were prepared and tested for activity with free cholesterol or egg yolk as the substrate (Table 2). All strains of *R. equi* degraded free cholesterol at a high rate. However, the rate of cholesterol degradation by cell-free extracts with egg yolk preparations was much lower. Some differences were seen among *Rhodococcus* strains in their ability to degrade egg yolk cholesterol (e.g. strains *R. equi* 33703 and 33706) even though rates of dissimilation of free cholesterol were similar (Table 2). In other cases (e.g. *R. equi* 21690), the rate of degradation for both free and egg yolk cholesterol was similar. A possible explanation for these results may be the more limited accessibility of cholesterol in egg yolk to the enzymes of the *Rhodococcus* dissimilation system. Alternatively, differences in activity levels among strains may be due to the presence or absence of additional enzyme systems (esterase, phospholipase) contributing to the general destabilization of cholesterol-

TABLE 2. *Effect of substrate on rates of cholesterol degradation by enzyme extracts of* Rhodococci.

Organism	Reaction rate (μg C degraded/min/mg protein) Free cholesterol[a]	Egg yolk cholesterol[a]
R. equi ATCC 33703	5.92	0.88
R. equi ATCC 33705	9.23	1.72
R. equi ATCC 33706	5.53	1.78
R. equi ATCC 21107	9.44	1.91
R. equi ATCC 6939	3.24	0.48
R. equi ATCC 21690	1.70	1.12
R. equi ATCC 7698	3.91	1.57
R. equi ATCC 7699	2.55	0.80

[a]Reaction conditions are described in Materials and Methods.

binding structures in egg yolk, and thus resulting in greater substrate availability. Extracts of strain *R. equi* 33706 degraded up to 40% of the egg yolk cholesterol after 60 min of incubation.

Data obtained on the degradation of cholesterol in the cream preparations by extracts of *R. equi* 33706 showed that only 2.36% of the cholesterol was removed after 1 h and 7.04% after 15 h of incubation. Apparently, the cholesterol in the cream preparations was even less accessible to the cholesterol dissimilating system of rhodococci than cholesterol in egg yolk preparations.

Properties of cholesterol degrading activity

The effect of temperature and pH on the cholesterol degrading activity of the rhodococci was studied using sonicated extracts of strain *R. equi* 33706. The optimum temperature of cholesterol degradation was approximately 45°C (Fig. 3). The cholesterol degrading system remained relatively stable following exposure to 60°C. After 1 h at 60°C approximately 44% of the initial activity was lost (Fig. 4).

The effect of pH on cholesterol dissimilation by *Rhodococcus* cell extracts is shown in Fig. 5. No activity was measurable at pH 4.0 and cholesterol dissimilation was at an optimum at pH 8.0.

Cholesterol dissimilation was not effected by the presence of Mg^{2+} or Ca^{2+} and the ineffectiveness of EDTA indicated the lack of divalent metal ion requirement for activity. However, the addition of either Zn^{2+} or ß-mercaptoethanol to the reaction mixture decreased the initial activity by 50% (Table 3).

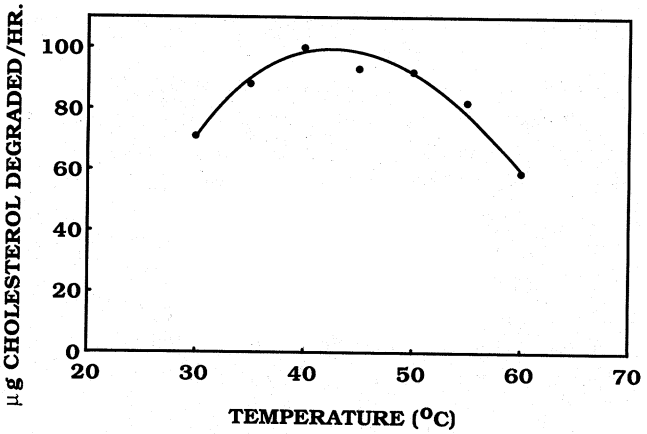

Figure 3. *Effect of temperature on cholesterol degradation by* R. equi *33706. Reaction conditions: Enzyme extract and substrate in 50 mM phosphate buffer (pH 7.4) were incubated at the temperature indicated for 60 min and extracted with ethyl acetate. Cholesterol content was determined with the ferric chloride reagent.*

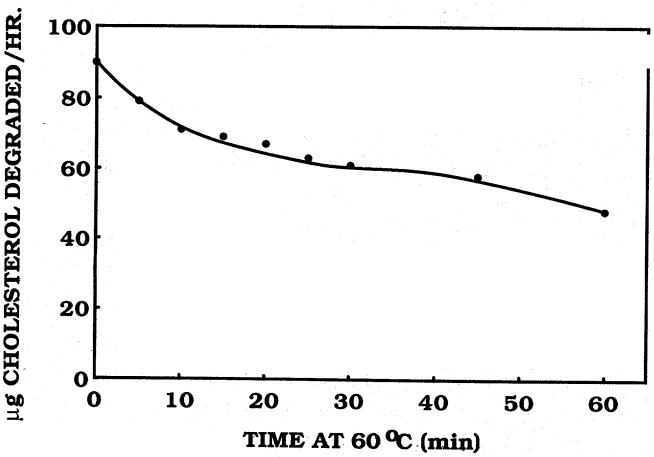

Figure 4. *Effect of heat treatment at 60°C on cholesterol degradation. Reaction conditions: Enzyme extracts were held at 60°C for varying lengths of time and residual activity was measured under standard conditions.*

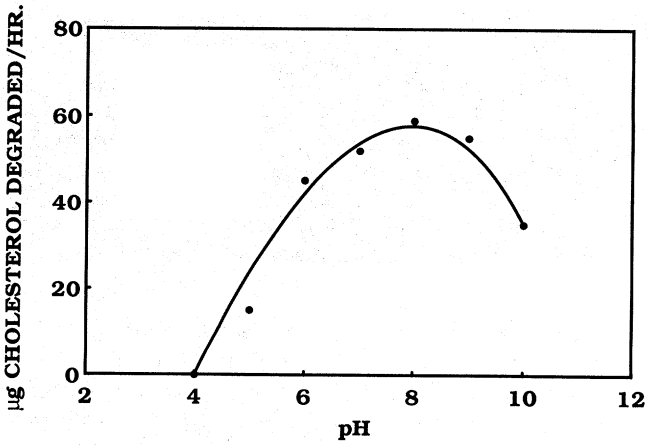

Figure 5. *Effect of pH on cholesterol degradation by* R. equi *33706. Enzyme assays were carried out in 100 mM acetate (pH 4 and 5), 100 mM phosphate (pH 6 and 7), and 100 mM Tris/HCl (pH 8 - 10) buffers at 37°C for 60 min with free cholesterol as substrate.*

TABLE 3. *Effect of EDTA, metal ions and sulfhydryl reagents on cholesterol degradation by* Rhodococcus equi *ATCC 33706 extracts.*

	Concentration (mM)	Percentage of initial activity[a] Nondialyzed	Dialyzed
CA^{2+}	10	103.1	98.6
Mg^{2+}	10	98.2	100.5
Zn^{2+}	10	46.2	50.8
EDTA	10	104.9	100.1
Mercaptoethanol	70	58.1	

[a]Activity of the extract in phosphate buffer without any additives.

The general properties of the *R. equi* cholesterol dissimilation system are similar to that reported in earlier studies on cholesterol oxidase, the first enzyme involved in the oxidative degradation of cholesterol. Cholesterol oxidases which catalyze the transformation of cholesterol to 4-cholesten-3-one have been described as active over wide ranges of pH and temperature and having no specific metal ion cofactor requirement, as in *Nocardia* sp (*10*), *N. rhodochrous* (3), *Streptomyces violascens* (12), *Streptoverticillium cholesterolicum* (*5*) and *Brevibacterium sterolicum* (*13*).

The results of our studies indicate that the *Rhodococcus* cholesterol degrading system is highly effective with free cholesterol but much less so with cholesterol in complex food systems such as egg yolk and milk cream. Further progress in reducing the cholesterol content of complex food systems will depend on the discovery of more efficient microbial enzymes and the development of procedures resulting in greater substrate availability to enzymatic degradation. However, at the present, the use of cholesterol degrading enzymes in foods remains unrealistic for lack of toxicological data on both enzyme extracts and intermediate products of cholesterol degradation.

ACKNOWLEDGMENT

The authors thank T. Terracino for able technical assistance.

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